THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 29, Issue of July 22, pp. 26896–26903, 2005
Printed in U.S.A.

Endothelin-induced, Long Lasting, and Ca^{2+} Influx-independent Blockade of Intrinsic Secretion in Pituitary Cells by G_z Subunits*

Received for publication, February 28, 2005, and in revised form, May 9, 2005
Published, JBC Papers in Press, May 26, 2005, DOI 10.1074/jbc.M502226200

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The G protein-coupled receptors in excitable cells have prominent roles in controlling Ca^{2+}-triggered secretion by modulating voltage-gated Ca^{2+} influx. In pituitary lactotrophs, spontaneous voltage-gated Ca^{2+} influx is sufficient to maintain prolactin release high. Here we show that endothelin in picomolar concentrations can interrupt such release for several hours downstream of spontaneous and high K^+-stimulated voltage-gated Ca^{2+} influx. This action occurred through the G_z signaling pathway; the adenylyl cyclase-signaling cascade could mediate sustained inhibition of secretion, whereas rapid inhibition also occurred at elevated cAMP levels regardless of the status of phospholipase C, tyrosine kinases, and protein kinase C. In a nanomolar concentration range, endothelin also inhibited voltage-gated Ca^{2+} influx through the $G_{i/o}$ signaling pathway. Thus, the coupling of seven-transmembrane domain endothelin receptors to G_z proteins provided a pathway that effectively blocked hormone secretion distal to Ca^{2+} entry, whereas the cross-coupling to $G_{i/o}$ proteins reinforced such inhibition by simultaneously reducing the pacemaking activity.

Calcium is the primary intracellular signaling molecule controlling the fusion of secretory vesicles with the plasma membrane to release transmitters from neurons and hormones from endocrine cells (1). This process is termed regulated exocytosis and is mediated by complex protein machinery that is conserved in organisms ranging from yeast to mammals. These proteins participate in docking, ATP-dependent priming, and fusion of vesicle membranes through interactions that are still not fully characterized (2–4). In regulated exocytosis, an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is required for two steps: priming the secretory vesicles, which occurs on the time scale of tens of seconds; and triggering the fusion, which occurs within 1–2 s (1). In excitable cells, both the depolarization-driven Ca^{2+} entry (5) and the agonist-induced Ca^{2+} mobilization from intracellular stores (6) can trigger the fusion of primed secretory vesicles.

The intracellular messenger cascades involving G proteins (7) have prominent effects on secretion, predominantly by modulating voltage-gated Ca^{2+} influx (VGCI)[1] and Ca^{2+} mobilization (8–12). Other intracellular messengers triggered by G protein-coupled receptors, including cAMP/protein kinase A (13, 14), diacylglycerol/protein kinase C (15–17), and phosphatidylinositol 4,5-bisphosphate/phosphatidylinositol 3-kinase (4, 18), can influence Ca^{2+}-triggered secretion by regulating the size of the releasable secretory pool and the rate of exocytosis. Furthermore, it has been suggested that G proteins may inhibit synaptic transmission in neuromuscular junction downstream of Ca^{2+} entry mechanisms (19, 20). It appears that the Ca^{2+}-independent inhibition of neurotransmission occurs through $G\beta\gamma$ subunits and their binding partners, syntaxin 1B and SNAP25B (21). At the present time, however, the identity of G proteins and role of $G\alpha$ subunits associated with $G\beta\gamma$ subunits responsible for such inhibition are unknown. The potential relevance of G subunits in inhibiting the fusion of dense core vesicles and the duration of such a blockade during the sustained firing of action potentials have also not been clarified.

Here we studied the relevance of G proteins in controlling the VGCI-triggered exocytosis of dense core vesicles in spontaneously firing pituitary lactotrophs. In these cells, the action potential-dependent fluctuations in $[Ca^{2+}]_i$ account for high basal prolactin (PRL) release (22). Such an intrinsic PRL release is controlled *in vivo* by three types of $G_{i/o}$ protein-coupled receptors: dopamine, somatostatin, and endothelin (ET) (11). Parallelism in the actions of ET-1 in a nanomolar concentration range on Ca^{2+} signaling and secretion in pituitary lactotrophs (23) and somatotrophs (24) suggests that the rate of exocytosis in these cells reflects the pattern of Ca^{2+} signaling. Our present results, however, indicate that ET receptors can inhibit PRL release independently of the status of Ca^{2+} signaling. We also present evidence that ET-1 desensitizes Ca^{2+} secretion coupling through G_z signaling pathway.

MATERIALS AND METHODS

Cell Cultures—Experiments were performed on anterior pituitary cells from normal postpubertal female Sprague-Dawley rats obtained from Taconic Farm (Germantown, NY). Pituitary cells were dispersed and cultured as mixed cells or enriched lactotrophs in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). A two-stage Percoll discontinuous density gradient procedure was used to obtain enriched lactotrophs, and further identification of lactotrophs in single cell studies was done by the addition of thyrotropin-releasing hormone (25).

PRL and cAMP Measurements—Cells (1 million/well) were plated in

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[1] The abbreviations used are: VGCI, voltage-gated Ca^{2+} influx; PRL, prolactin; ET, endothelin; PTX, pertussis toxin; PMA, phorbol 12-myristate 13-acetate.

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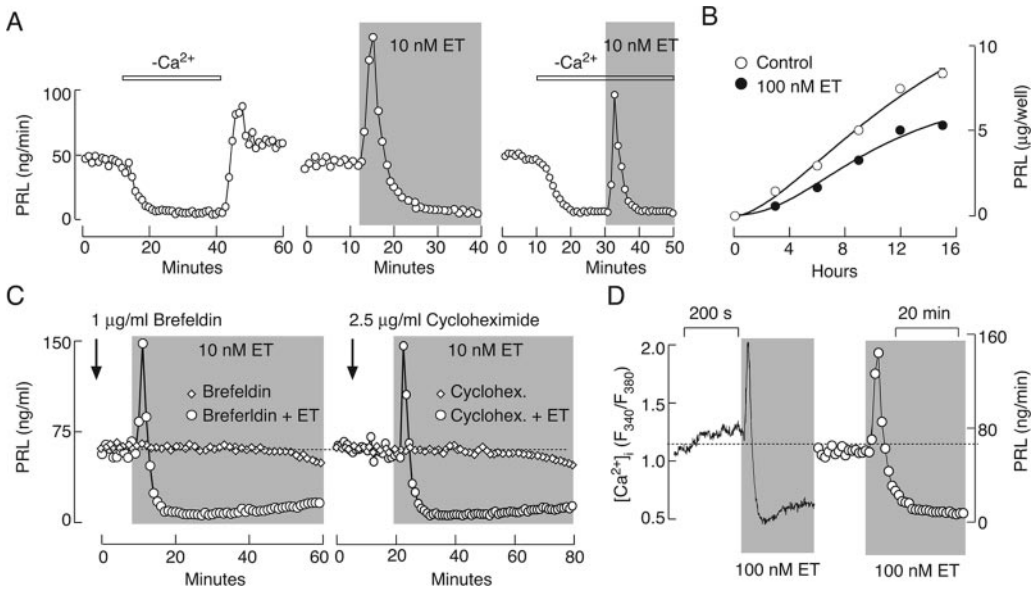

FIG. 1. **Characterization of ET-induced Ca^{2+} signaling and secretion in pituitary lactotrophs.** *A*, *left*, extracellular calcium dependence of basal PRL release in perifused pituitary cells; representative trace from eight experiments. *A*, *middle*, time course of ET-induced PRL release in pituitary cells perifused with Ca^{2+}-containing medium, representative trace from 36 experiments. *A*, *right*, time course of ET-induced PRL release in pituitary cells perifused with Ca^{2+}-deficient medium, representative trace from three experiments. *B*, time course of PRL release in static pituitary cells in the presence and absence of ET-1 (mean values with $n = 6$; S.E. are within *circles*). *C*, the lack of effect of inhibitors of protein synthesis, brefeldin and cycloheximide (*Cyclohex.*), on ET-induced PRL release. *D*, bidirectional effects of ET-1 on calcium signaling (*left*) and PRL release (*right*). The averaged Ca^{2+} profile was obtained from 15 single lactotrophs, and the secretory profile was generated from five perifusion experiments. In this and the following figures, *gray areas* indicate the duration of ET-1 application. Secretory studies were done in unpurified cells, and Ca^{2+} measurements were done in single identified lactotrophs.

24-well plates in serum-containing M199 and incubated overnight at 37 °C under 5% CO_2-air and saturated humidity. Prior to the experiments, cells were washed with serum-free medium and stimulated at 37 °C under 5% CO_2-air and saturated humidity for 120 min if not otherwise stated. Hormone secretion was also monitored using cell column perifusion experiments. Briefly, 1.2×10^7 cells were incubated with preswollen Cytodex-1 beads in 60-mm Petri dishes for 24 h. The beads were then transferred to 0.5-ml chambers and perifused with Hanks' M199 containing 25 mM HEPES, 0.1% bovine serum albumin, and penicillin (100 units/ml)/streptomycin (100 μg/ml) for 2.5 h at a flow rate of 0.8 ml/min and at 37 °C to establish stable basal secretion. Fractions were collected at 1-min intervals, stored at −20 °C, and later assayed for PRL and cAMP contents using radioimmunoassay. Primary antibody and standard for PRL assay were provided by the National Pituitary Agency and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA); ^{125}I-labeled PRL was purchased from PerkinElmer Life Sciences and secondary antibody from Sigma. Cyclic AMP was determined in both media and cell contents using specific antiserum provided by Albert Baukal (NICHD, National Institutes of Health, Bethesda, MD).

Single Cell Calcium Measurements—For $[Ca^{2+}]_i$ measurements, cells were incubated in Hanks' M199 supplemented with 2 μM fura-2 AM (Molecular Probes, Eugene OR) at 37 °C for 60 min. Coverslips with cells were then washed and mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor digital fluorescence microscopy system (Atto Instruments, Rockville, MD). Cells were examined under a 40× oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 520 nm was measured. The ratio of light intensities, F_{340}/F_{380}, which reflects changes in Ca^{2+} concentration, was followed in several single cells simultaneously at the rate of 1 point/s. The $[Ca^{2+}]_i$ was calibrated as described previously (22).

Western Blot Analysis—Crude membranes were prepared as described previously (26). Protein concentration was estimated by the Bradford method with bovine serum albumin as a standard (Pierce). Equal amounts of proteins were run on one-dimensional SDS-PAGE using a discontinuous buffer system (Novex), and proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) with a wet transfer and following the manufacturer's recommendation. The immunodetection of G proteins was done with G_z, G_s (Santa Cruz Biotechnology, Santa Cruz, CA), G_q, G_{i1}–G_{i3}, G_o, G_{12}, and G_{13} (Calbiochem) α-protein-specific antibodies. β-Actin was detected using monoclonal antibody produced by Oncogene Research Products (San Diego, CA). The secondary antibodies were a goat anti-rabbit IgG or anti-mouse IgG-IgM (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Secondary antibodies were linked to horseradish peroxidase. The reactive bands were always determined with a luminol-based kit (Pierce), and the reaction was detected by an enhanced chemiluminescence system using x-ray film.

G_z Antisense Experiments—The oligodeoxynucleotides were from Invitrogen. The sequence of $G_z\alpha$ antisense oligodeoxynucleotide was 5′-CGTGATCTCACCCTTGCTCTCTGCCGGGCT-3′, whereas the sequence of the missense oligodeoxynucleotide was 5′-CCCTTATTTACTTTCGCC-3′ (27). Both sequences were phosphorothioate-modified only at positions 5′-CC and GC-3′ (28). Oligodeoxynucleotides were dissolved in 1× TE buffer, containing 10 mM Tris, pH 8.0, and 0.1 mM EDTA, according to the recommendation of the producer. A purified lactotroph fraction of cells were plated in 24-well plates (10^6 cells/ml/well) and cultured for 6 h in culture medium enriched with 10% horse serum. Then the medium was replaced, and 0.25 nmol of antisense or missense oligodeoxynucleotides in 2 μl of TE buffer/well was applied every hour during the following 36-h time period, whereas the cells in control wells received an equal volume of TE buffer alone. After that medium was changed, the procedure was repeated for an additional 36 h.

Calculations—The time course of PRL release was fitted to a single exponential function ($ae^{-kt} + b$) using GraphPad Prism (GraphPad Software, Inc., San Diego, CA) to generate the rates of signaling desensitization (k). Concentration-response relationships were fitted to a four-parameter logistic equation using a nonlinear curve-fitting program, which derives 50% efficient concentrations (EC_{50}) and 50% inhibitory concentrations (IC_{50}) (Kaleidagraph, Synergy Software Technologies, Reading, PA). In the figures, the results shown are means ± S.E. from sextuplicate determination in one of at least three similar experiments, and asterisks indicate a significant difference ($p < 0.01$) among means, estimated by Student's t test.

RESULTS AND DISCUSSION

ET Inhibits Extracellular Ca^{2+}-dependent Basal PRL Release—Lactotrophs exhibit spontaneous firing of action potentials; the associated Ca^{2+} transients are sufficient to maintain PRL release at high and steady levels in cells in population for many hours (22). Such secretion is termed intrinsic, spontane-

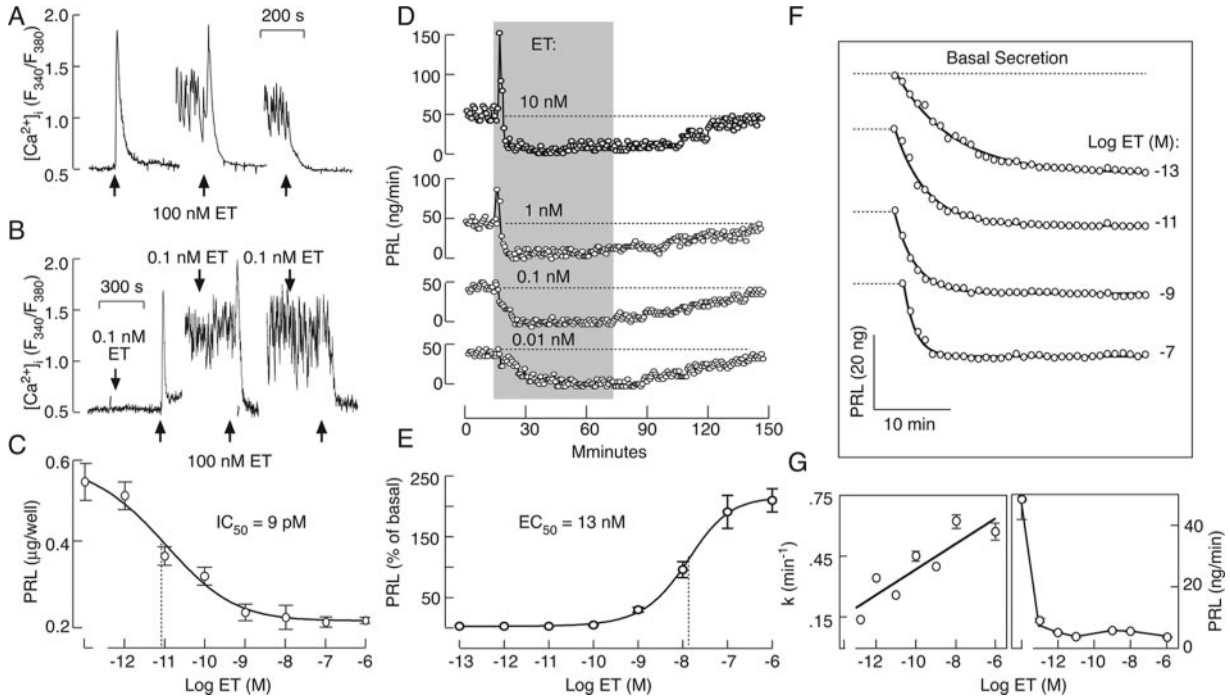

FIG. 2. **Dissociation between Ca^{2+} signaling and secretion in ET-stimulated lactotrophs.** *A*, typical patterns of ET-induced changes in $[Ca^{2+}]_i$ in single lactotrophs: monophasic response in quiescent cells (*left*; 42 of 95 cells), bidirectional response in spontaneously active cells (*middle*; 44 of 95 cells), and inhibition of Ca^{2+} transients without triggering calcium mobilization (*right*; 9 of 95 cells). *B*, the lack of effect of subnanomolar ET-1 on $[Ca^{2+}]_i$ in single lactotrophs; representative traces from at least 10 cells/group. *C*, dose-dependent effects of ET-1 on PRL release in cells in static cultures incubated for 2 h. *D*, PRL secretory profiles in cells perifused with variable concentrations of ET-1. *E*, dose-dependent effects of ET-1 on spike PRL release. *F*, dose-dependent effects of ET-1 on the rates of inhibition of PRL release in cell perifusion experiments. A mono-exponential function (*solid lines*) was used to fit experimental data (*open circles*). For clarity, data showing stimulatory effects of ET-1 have been removed, and basal PRL release prior to agonist application is indicated by *dotted lines*. *G*, dose-dependent effects of ET-1 on rates (*left*) and levels (*right*) of PRL inhibition. *Vertical dotted lines* illustrate the calculated IC_{50} and EC_{50} values. Data shown are means ± S.E. from sextuplicate incubations in one of four similar experiments (*C*) and means ± S.E. from three independent experiments (*E* and *G*). All of the experiments were done with unpurified pituitary cells.

ous, or basal (12). Fig. 1*A*, *left*, illustrates the extracellular Ca^{2+} dependence of basal PRL release in pituitary cells perifused at a flow rate of 0.8 ml/min. Perifusion chambers containing 12×10^6 cells (with an estimated number of lactotrophs of 2×10^6) released between 30 and 90 ng of PRL/min (mean value from 40 experiments = 55.2 ± 4.4 ng/min), corresponding to ~15–45 fg/min PRL released per cell. Depletion of extracellular Ca^{2+} induced a decrease in PRL release to 6.3 ± 0.8 ng/ml (n = 8), and the reconstitution of Ca^{2+} led to a full recovery of secretion with a transient overshot (Fig. 1*A*). There was also a progressive accumulation of PRL in the medium during a 16-h incubation of cells in static cultures (Fig. 1*B*). The estimated rate of release per cell ranged between 20 and 40 fg/min. Basal PRL secretion was not immediately affected by the inhibition of *de novo* PRL synthesis, as documented in experiments with the application of brefeldin and cycloheximide (Fig. 1*C*).

Spontaneous PRL release may be enhanced by the $G_{q/11}$-coupled thyrotropin-releasing hormone and angiotensin II receptors (9, 23) and inhibited by $G_{i/o}$-coupled dopamine and somatostatin receptors (11). G protein-coupled ET receptors (29) are also present in pituitary cells and participate in the control of secretion (30). Several reports suggest that the ET-A subtype of these receptors is expressed in anterior pituitary cells (24, 31–33), whereas the ET-B receptor subtype may be expressed in the intermediate lobe of the gland (34).

The activation of ET receptors in lactotrophs mimics the actions of both $G_{q/11}$ and $G_{i/o}$ protein-coupled receptors on Ca^{2+} signaling and PRL secretion (23, 35–39). Perifusion experiments revealed the presence of two phases in the action of ET-1 on PRL release: a rapid and transient increase in PRL release and a sustained decrease in secretion below the basal levels (Fig. 1*A*, *center*; hereafter the bidirectional response). As in cells perifused with Ca^{2+}-deficient medium, PRL secretion dropped to 5.9 ± 0.7 ng/ml (n = 36) during the sustained ET-1 application, clearly showing that this agonist blocks Ca^{2+} influx-dependent PRL release. In cells perifused with Ca^{2+}-deficient medium, ET induced only monophasic response (Fig. 1*A*, *right*). In cells in static cultures, the application of 100 nM ET-1 induced a long lasting inhibition of PRL release, whereas the stimulatory effect was not visible (Fig. 1*B*).

The ET-induced decrease in PRL release was not related to the status of PRL synthesis or the depletion of the secretory pool (Fig. 1*C*). Our previous work (23) shows the parallelism in the actions of 100 nM ET-1 on $[Ca^{2+}]_i$ and PRL secretion. Fig. 1*D* illustrates such an example. The Ca^{2+} trace shown represents the mean value from 15 single lactotrophs, and the secretory profile was generated from 5 perifusion experiments. Both the extracellular Ca^{2+} dependence of PRL release (Fig. 1*A*) and the bidirectional effects of ET-1 on Ca^{2+} and PRL release (Fig. 1*D*) are consistent with the hypothesis that the rate of PRL release in these cells reflects changes in $[Ca^{2+}]_i$.

ET Inhibits Basal PRL Release Downstream of VGCI—Single cell Ca^{2+} analysis showed that the pattern of ET-1-induced Ca^{2+} response varies among cells. In the majority of spontaneously active lactotrophs, $[Ca^{2+}]_i$ fluctuated between 0.7 and 1.5 values when expressed as F_{340}/F_{380} with a mean value of 0.94 ± 0.08 (n = 21), which corresponds to about 350 nM. 100 nM ET-1 induced an initial spike increase in $[Ca^{2+}]_i$ and a sustained decrease in $[Ca^{2+}]_i$ below the resting level (Fig. 2*A*, *center*). The mean value of $[Ca^{2+}]_i$ during the sustained ET application was 0.63 ± 0.06 (n = 35), which corresponded to about 100 nM. Depletion of extracellular Ca^{2+} also decreased

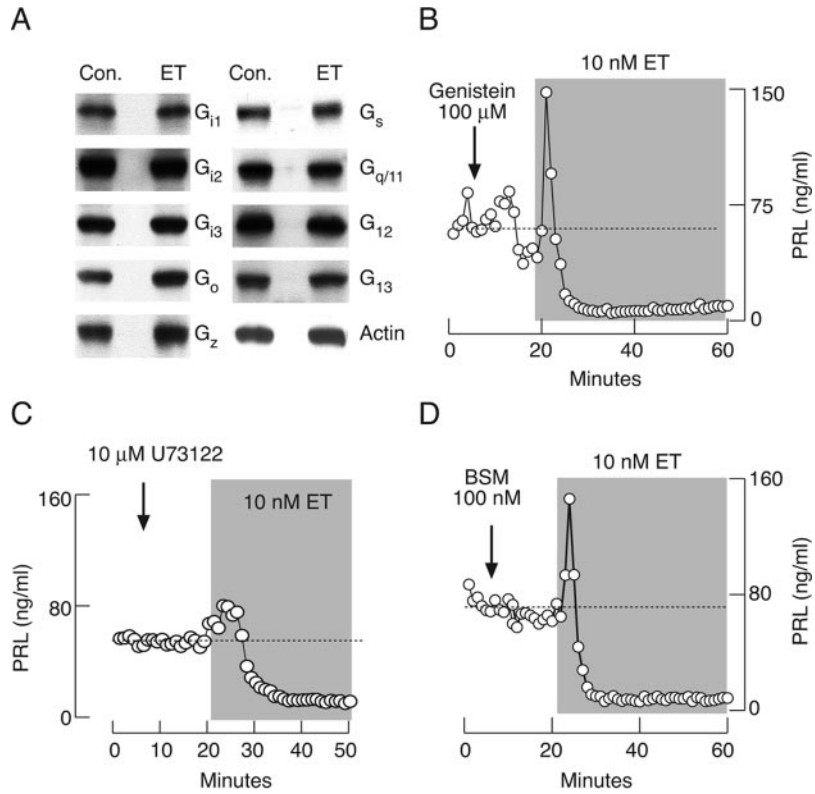

FIG. 3. **Independence of ET-induced inhibition of PRL release of phospholipase C and tyrosine kinases.** *A*, expression of Gα proteins in pituitary cells. Western blot analysis was done in membrane fractions from controls (*Con.*) and pituitary cells treated with 100 nM ET-1 for 20 min. *B*, the lack of effect of genistein, a tyrosine kinase inhibitor, on ET-1-induced PRL release. *C*, effects of U73122 (10 μM), a phospholipase C inhibitor, on spike response. *D*, the lack of effect of bisindolylmaleimide (*BSM*), a protein kinase C inhibitor, on ET-1-induced PRL release. All of the experiments were done with unpurified pituitary cells. Traces shown are representative of three similar experiments.

$[Ca^{2+}]_i$ to comparable levels (F_{340}/F_{380} = 0.62 ± 0.05; n = 21). In some cells, only the second phase was observed (Fig. 2*A*, *right*). In silent cells, the mean F_{340}/F_{380} value of $[Ca^{2+}]_i$ (0.67 ± 0.05, n = 14) was highly comparable with that observed in ET-stimulated cells during the sustained application. In such cells, ET induced a monophasic stimulatory response (Fig. 2*A*, *left*). The transient spike phase was observed upon ET-1 stimulation with concentrations of 1 nM or higher but with variable occurrences: 71% (24 of 34) of the cells responded to 100 nM, 28% (9 of 32) of the cells responded to 10 nM, and 9% (3 of 34) of the cells responded to 1 nM. The spike response was not observed in any cells stimulated with 0.1 nM (Fig. 2*B*) and lower ET-1 concentrations. The inhibition of spontaneous VGCI also occurred in the 1–100 nM ET-1 concentration range. For example, Ca^{2+} transients were stopped in 56% (19 of 34) of the cells stimulated with 100 nM ET-1, in 25% (8 of 32) of the cells stimulated with 10 nM ET-1, and in none of the cells stimulated with 0.1 nM (Fig. 2*B*) and lower concentrations. The lack of effect of picomolar concentrations of ET on $[Ca^{2+}]_i$ was independent of the duration of agonist application, as no changes in the pattern of signals in spontaneously active cells were observed during a 30-min incubation (mean value of $[Ca^{2+}]_i$, 0.93 ± 0.07 or about 350 nM, n = 17). The roughly estimated EC_{50} and IC_{50} values were on the order of 10 nM in magnitude.

In contrast to Ca^{2+}, ET-1 inhibited PRL release in static cultures with a calculated IC_{50} of 9 pM (Fig. 2*C*), a thousand-fold leftward shift in the potency of ET-1 to stop spontaneous Ca^{2+} transients. In perifused pituitary cells, ET-1 was also able to inhibit PRL release when added in 0.1 and 0.01 nM concentrations, whereas the spike PRL response was observed only if the concentration of ET-1 exceeded 0.1 nM (Fig. 2*D*), with a calculated EC_{50} of 13 nM (Fig. 2*E*). The detailed kinetic analysis of PRL secretion (Fig. 2*F*) revealed that the rate of inhibition increased with the elevation of agonist concentration (Fig. 2*G*, *left*). On the other hand, the plateau levels of inhibition after 20 min of stimulation were comparable in a 1 pM to 1 μM concentration range of ET-1 (Fig. 2*G*, *right*), amounting to 6.2 ± 0.7 ng/ml (n = 12) during the sustained application of 10 nM and, when stimulated with 100 pM, to 6.8 ± 0.9 ng/ml (n = 6). In cells perifused with ET-1 for longer than 30 min, there was a gradual recovery in PRL release, and the rates of this recovery inversely correlated with agonist concentrations (data not shown). These studies indicate that in the picomolar concentration range ET-induced blockade of PRL secretion is independent of the VGCI status.

ET Inhibits PRL Release in a Phospholipase C- and Tyrosine Kinase-independent Manner—Pituitary cells express the full repertoire of Gα proteins (Fig. 3*A*). In contrast to other cell types (26, 40), the application of agonist in pituitary cells did not decrease the plasma membrane localization of any of the α subunits, indicating that this method cannot be used to identify the G proteins activated by ET receptors (Fig. 3*A*). The agonist-induced blockade of PRL release was not affected in cells in which tyrosine kinases were inhibited by genistein (Fig. 3*B*). Previous studies showed the coupling of pituitary ET receptors to $G_{q/11}$ and activation of phospholipase C, leading to an increase in inositol 1,4,5-triphosphate production and subsequently to Ca^{2+} mobilization from intracellular stores (23, 41). Consistent with these findings, in cells treated with U73122, a phospholipase C inhibitor, the spike phase of ET-induced PRL release was reduced, but the inhibitory phase was not affected (Fig. 3*C*). Furthermore, the ET-induced bidirectional pattern of PRL release was not changed in cells with protein kinase C inhibited by bisindolylmaleimide (Fig. 3*D*). Thus, the coupling of ET receptors to the phospholipase C signaling pathway is not responsible for sustained inhibition of basal PRL release.

Coupling of ET Receptors to $G_{i/o}$ Proteins—Experiments with the pertussis toxin (PTX) indicated that the coupling of ET receptors to $G_{i/o}$ proteins provides an effective mechanism for the inhibition of spontaneous VGCI by activating inwardly rectifying K^+ channels (39). As shown in Fig. 4*A*, the bidirectional response of $[Ca^{2+}]_i$ typically observed in control cells during the application of ET-1 (*top left*) was replaced with a biphasic response in cells treated overnight with PTX, which

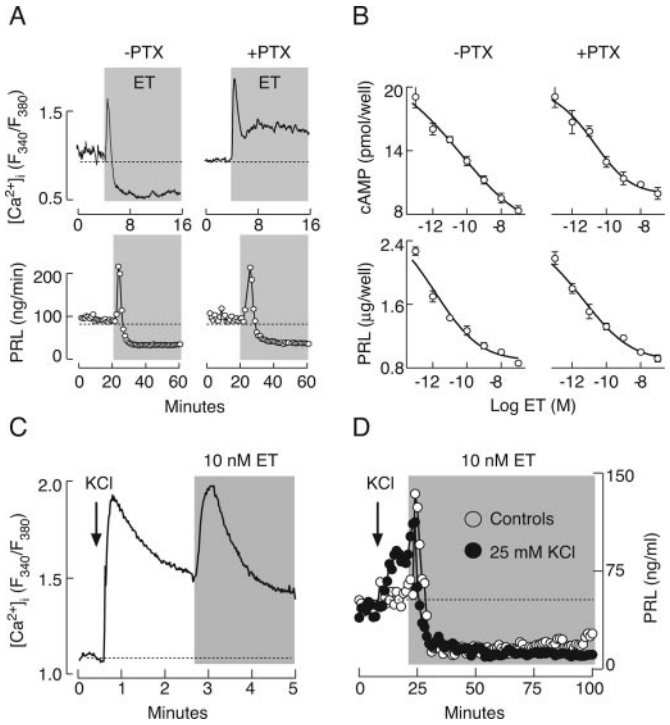

FIG. 4. **Independence of ET-induced inhibition of PRL release of $G_{i/o}$ coupling.** *A*, patterns of ET-induced Ca^{2+} signaling (*top*) and PRL secretion (*bottom*) in controls (*left*) and cells pretreated overnight with 250 ng/ml PTX (*right*). Ca^{2+} traces shown are means from 32 records (*left*) and 35 records (*right*). For PRL, results shown are means from five columns (*left*) and eight columns (*right*). *B*, dose-dependent effects of ET-1 on cAMP accumulation (*top*) and PRL release (*bottom*) in controls (*left*) and PTX-treated (*right*) pituitary cells in static culture are indicated. Cells were treated overnight with 250 ng/ml PTX, and cAMP levels were measured in both cell extracts (content) and medium (released) and expressed as combined values (mean ± S.E., $n = 6$). PRL was measured in medium from the same samples. Endogenous phosphodiesterase activity was attenuated by the addition of 1 mM 3-isobutyl-1-methylxanthine. *C* and *D*, characterization of effects of ET-1 on $[Ca^{2+}]_i$ (*C*) and PRL release (*D*) in high K^+-depolarized cells. The Ca^{2+} trace represents the mean from 14 individual cells. Experiments were done in unpurified cells (*A*, *bottom*, and *D*) or purified lactotrophs (*B*), and Ca^{2+} measurements were done with single identified lactotrophs (*A*, *top*, and *C*).

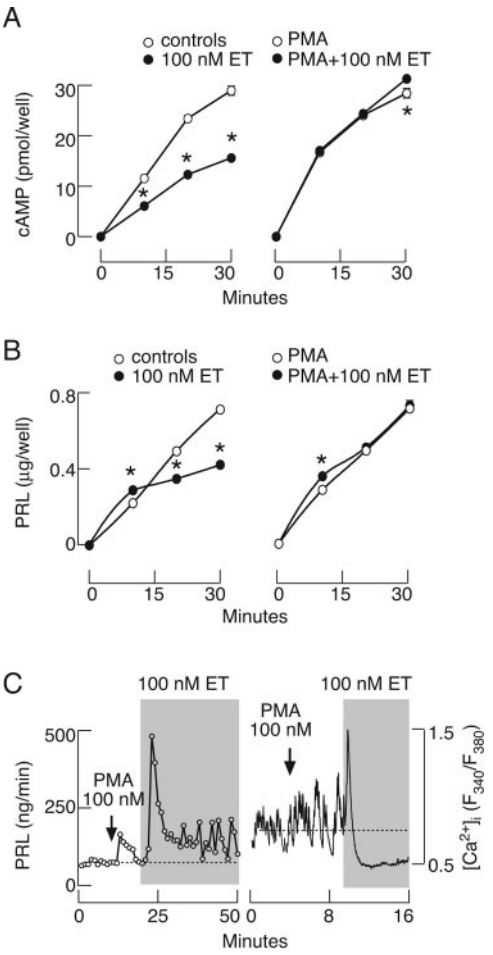

FIG. 5. **Coupling of ET receptors to G_z signaling pathway in pituitary cells.** Effect of phorbol ester (PMA) on 100 nM ET-induced cAMP/Ca^{2+} signaling and PRL release. Time course of ET-1 (100 nM) effects on cAMP accumulation (*A*) and PRL release (*B*) in control cells (*left*) and PMA (100 nM)-treated cells (*right*) in static culture are indicated. *Asterisks* indicate significant differences between pairs (mean values ± S.E.; $n = 6$). The endogenous phosphodiesterase activity was attenuated by 1 mM 3-isobutyl-1-methylxanthine. *C*, effects of PMA on ET-induced Ca^{2+} signaling (*right*) and PRL release (*left*) in perifused pituitary cells; representative traces from four experiments (PRL) and 18 cells ($[Ca^{2+}]_i$). All of the experiments were done using purified lactotrophs, and Ca^{2+} measurements were done with identified cells.

was composed of an early spike phase and a sustained plateau phase of elevated $[Ca^{2+}]_i$ (*top right*). In contrast to Ca^{2+} signaling, the bidirectional pattern of ET-induced PRL secretion was not affected by PTX treatment, and the levels of sustained secretion inhibition were comparable between controls and PTX-treated cells (Fig. 4*A*, *bottom*). The inhibition of PRL release by ET-1 was also observed in PTX-treated static cultures of pituitary cells (Fig. 4*B*, *bottom*). Furthermore, the ET-1 was unable to decrease $[Ca^{2+}]_i$ in high K^+-depolarized cells but decreased PRL release to the levels observed in control cells (Fig. 4, *C* and *D*). Thus, the ET-induced blockade of PRL secretion is independent of the VGCI status, and the G proteins other than $G_{i/o}$ and $G_{q/11}$ play a role in stopping PRL release at elevated $[Ca^{2+}]_i$.

Coupling of ET Receptors to G_z Proteins—Interestingly, the coupling of pituitary ET receptors to the $G_{i/o}$ signaling pathway did not account for the attenuation of adenylyl cyclase activity. In control cells in a static culture, ET-1 decreased cAMP production in a dose-dependent manner with an IC_{50} of ~10 pM (Fig. 4*B*, *top left*) comparable with that observed for the inhibition of PRL release in the same experiment (*bottom left*). In cells treated overnight with PTX, the ET-induced attenuation of adenylyl cyclase activity (Fig. 4*B*, *top right*), as well as its blockade on PRL release (*bottom right*), was maintained. The lack of effect of PTX-treatment on ET-induced inhibition of cAMP production is consistent with published findings in other cell types, indicating a role of PTX-insensitive $G_z\alpha$ in control of adenylyl cyclase activity (42).

To prevent the coupling of ET receptors to G_z-dependent signaling pathways, we used two experimental approaches. In the first series of experiments, cells were stimulated with phorbol esters, which should silence the G_z signaling pathway through the protein kinase C-dependent phosphorylation of the $G_z\alpha$ subunits (43, 44). Pituitary cells in static cultures were treated with 100 nM ET-1 in the presence or absence of 100 nM phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 5*A*, *left*, cAMP accumulation in control cells was inhibited by ET-1 in a time-dependent manner. In PMA-treated cells, however, such inhibition was not observed, indicating that the negative coupling of ET receptors to adenylyl cyclase was lost (Fig. 5*A*, *right*). Parallel to the effects of ET-1 on cAMP, PRL release was inhibited during the sustained agonist application in control cells (Fig. 5*B*, *left*) but not in PMA-treated cells (*right*). In perifused pituitary cells, the ET-induced inhibition of PRL

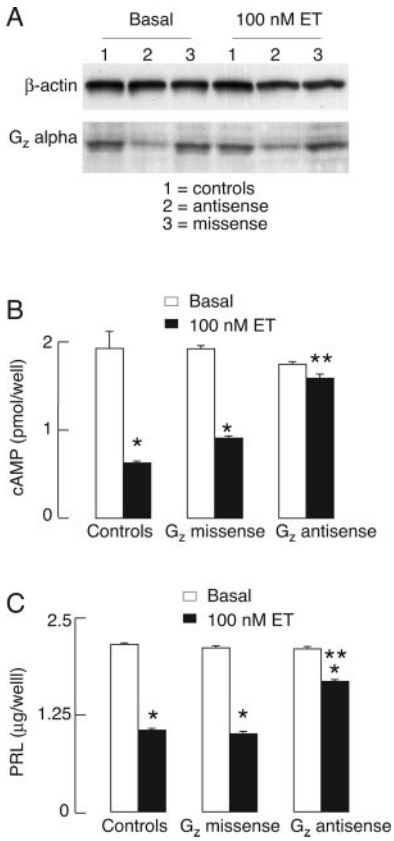

FIG. 6. **Effects of down-regulation of G_z expression in pituitary cells on ET-induced cAMP signaling and PRL release.** *A*, Western blot analysis of $G_z\alpha$ expression in membrane fractions from controls and cells treated with $G_z\alpha$-specific antisense and missense oligodeoxynucleotides. cAMP (*B*) and PRL release (*C*) in controls and pituitary cells exposed to antisense and missense oligodeoxynucleotides in either the absence (*open bars*) or the presence (*filled bars*) of 100 nM ET-1. After a 72-h treatment with oligodeoxynucleotides, cells were washed and stimulated with ET-1 for 2 h. cAMP was measured only in medium because the cell content was used for Western blot analysis. The endogenous phosphodiesterase activity was attenuated by 1 mM 3-isobutyl-1-methylxanthine. *, $p < 0.01$ *versus* basal secretion; **, $p < 0.01$ *versus* missense treatment. All of the experiments were done using purified lactotrophs.

release was abolished in the presence of PMA even though the pattern of Ca^{2+} signaling was not affected (Fig. 5*C*), although one might expect that the lowering of $[Ca^{2+}]_i$ by sustained application of ET would attenuate the underlying secretion rate. Note also the increased amplitude of the spike response in PMA-treated cells (Fig. 5*C*, *left*) compared with controls (Figs. 1–4), a finding consistent with the role of protein kinase C in the sensitization of Ca^{2+} secretion coupling (15–17).

The possibility that the activation of protein kinase C makes intrinsic secretion independent of or less dependent on extracellular Ca^{2+} influx prompted us to perform experiments with the down-regulation of $G_z\alpha$ expression in lactotrophs. For this purpose, we continuously treated cells for 72 h with specific antisense phosphorothio-modified oligodeoxynucleotides, whereas control cells were treated with missense phosphorothio-modified oligodeoxynucleotides. Treatment with $G_z\alpha$-specific antisense oligodeoxynucleotides but not missense oligodeoxynucleotides substantially decreased the integrated optical density of the $G_z\alpha$ protein band (Fig. 6*A*). The negative coupling of ET receptors to the adenylyl cyclase signaling pathway was practically abolished in antisense oligodeoxynucleotides-treated cells (Fig. 6*B*). In the same samples, the ET-induced decrease of PRL release was substantially reduced (Fig. 6*C*), confirming the involvement of a G_z signaling pathway in the inhibitory actions of ET receptors on Ca^{2+}-triggered exocytosis.

The finding that ET-1 inhibits both cAMP production and PRL release in lactotrophs may indicate a role for protein kinase A in controlling secretion, as observed in other cell types (14). To test this hypothesis, pituitary cells were perifused with forskolin, an activator of adenylyl cyclase. This treatment dramatically increased cAMP release (Fig. 7*A*, *top*). The application of 100 nM ET-1 to the cells before cAMP release reached the steady state immediately decreased the rate of release, indicating that ET-1 was able to attenuate adenylyl cyclase activity even in the presence of forskolin. However, the levels of cAMP in forskolin-treated cells during the sustained ET-1 stimulation were higher than those observed in controls (Fig. 7*A*, *top*). Measurements of PRL content in the same samples revealed an increase in secretion during the forskolin treatment. Furthermore, the application of 100 nM ET-1 in the presence of forskolin was followed by a decrease in PRL release. Finally, there was a rapid recovery of PRL release in forskolin-treated cells after the removal of ET-1, in contrast to control cells (Fig. 7*A*, *bottom*).

To exclude the possible nonspecific effects of forskolin on PRL secretion, the cell-permeable 8-cAMP analog, 8-bromo-cAMP, was used. Application of this compound mimicked the action of forskolin on basal PRL release. In addition, upon the application of ET-1 there was a sustained attenuation of PRL release, and removal of ET-1 was followed by a rapid recovery of PRL release above basal levels (Fig. 7*B*). The transient inhibitory effect of ET-1 on PRL release was also observed in forskolin-treated cells (Fig. 7*C*), in which agonist-induced inhibition of VGCI was blocked by overnight treatment with PTX (Fig. 4*A*), as well as in forskolin-treated cells during the application of 0.1 nM ET-1 (Fig. 7*D*), a concentration that does not affect spontaneous VGCI (Fig. 2*B*). Thus, although in these experimental conditions both cAMP and $[Ca^{2+}]_i$ were elevated, the inhibitory effect of ET-1 on PRL release was maintained. However, this effect was not as prominent as in control cells, and the recovery of secretion was initiated even in the presence of ET-1.

Concluding Remarks—Our results show that the coupling of receptors to $G_{i/o}$ proteins and the subsequent silence of pacemaker activity (39) through activation of inwardly rectifying K^+ channels and inhibition of voltage-gated Ca^{2+} channels (45, 46) are not sufficient to conclude that this particular pathway mediates the inhibition of secretion. Two lines of evidence support this conclusion. First, ET inhibits basal PRL secretion in picomolar concentrations, which do not affect spontaneous VGCI. Second, in cells with $G_{i/o}$ coupling blocked by PTX, ET inhibits secretion but not VGCI. These results indicate that ET inhibits PRL secretion through the intracellular mechanism that is downstream of VGCI. Thus, the G protein-mediated inhibition of secretion distal to Ca^{2+} influx is not unique for synaptic transmission (19–21) but also occurs in endocrine cells involving dense core vesicles.

Here we show for the first time that the heterotrimeric G_z proteins provide a pathway through which ET receptors can stop secretion for a prolonged period of time without preventing pacemaking activity. Again, two lines of evidence support this conclusion. First, experiments with phorbol esters, which silence the G_z signaling pathway through the protein kinase C-dependent phosphorylation of the $G_z\alpha$ subunits (43, 44), indicated that the inhibitory action of ET was lost. This finding, however, should be taken with reservation because of the complex actions of protein kinase C on exocytosis (15–17, 47). However, more conclusive results were obtained by down-regulating the G_z expression.

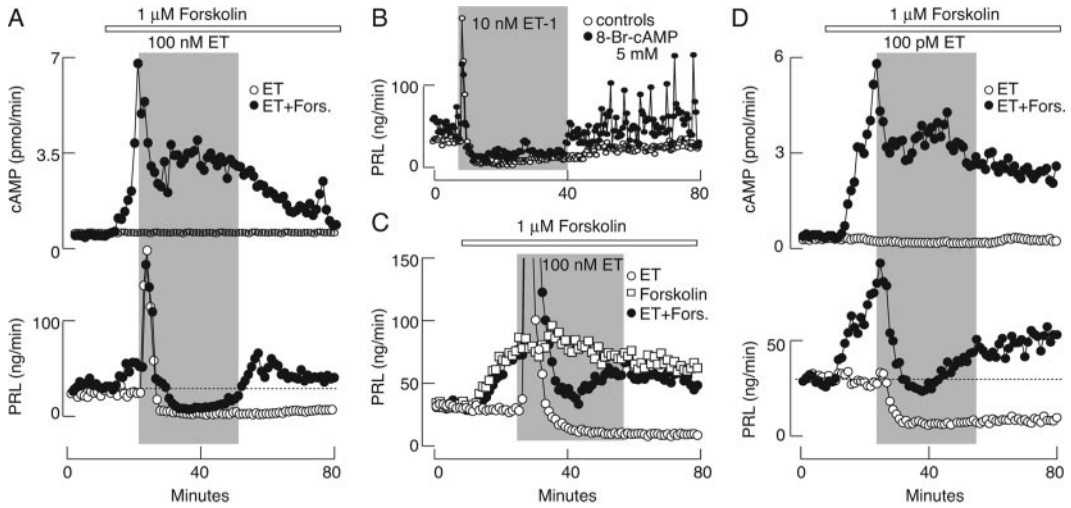

FIG. 7. **Dependence of ET-induced inhibition of PRL release on adenylyl cyclase activity.** *A* and *D*, effects of 100 nM (*A*) and 0.1 nM ET-1 (*D*) on cAMP (*top*) and PRL release (*bottom*) in forskolin (1 μM)-treated (*filled circles*) and untreated (*open circles*) perifused pituitary cells. *B*, ET-induced inhibition of PRL release in perifused pituitary cells in the presence of 5 mM 8-bromo-cAMP. *C*, effects of 1 μM forskolin on PRL release alone and in combination with 100 nM ET-1 in cells with blocked $G_{i/o}$ signaling pathway. All of the experiments were done with unpurified pituitary cells.

Our results are consistent with the participation of both arms of G_z proteins, α and $\beta\gamma$, in an ET-induced inhibition of PRL release downstream of VGCI. The $G_z\alpha$-mediated attenuation of adenylyl cyclase activity in pituitary cells may account for the sustained inhibition of PRL release during ET application and the slow recovery of secretion after agonist removal. In accordance with this, it has been shown recently that protein kinase A-dependent phosphorylation of SNAP25 increases the size of slowly and rapidly releasable secretory vesicle pools without affecting the kinetics of vesicle fusion (13, 14). However, to what extent the $G_z\alpha$-mediated inhibition of adenylyl cyclase in lactotrophs leads to depletion of the primed secretory vesicle pool during the sustained agonist stimulation in normal physiological situations (in cells without inhibited phosphodiesterases) needs further evaluation.

Furthermore, the rapid decrease in PRL release induced by ET-1 at basal and elevated cAMP levels, as well as the inhibition of PRL release in cells in static cultures with inhibited phosphodiesterases, suggests the role of $G_z\beta\gamma$ in controlling exocytosis. This conclusion is in accordance with the findings in other cell types about the role of $G\beta\gamma$ subunits in control of secretion independently of their actions on adenylyl cyclase, phospholipase C-β2, and several tyrosine kinases (21). Recent experiments by Martin and co-workers (48) in permeabilized PC12 cells demonstrated that G protein $\beta\gamma$ directly regulates soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein fusion machinery for secretory granule exocytosis. This action occurs rapidly and affects ATP-primed secretory vesicles. The C terminus of SNAP25, which links synaptotagmin I to the SNARE complex, may represent a target of $G\beta\gamma$ for such inhibition (48, 49).

Therefore, it is reasonable to suggest that the liberation of $G_z\beta\gamma$ represents the primary factor in ET-induced inhibition of VGCI-dependent PRL secretion, whereas the liberation of $G_{i/o}\beta\gamma$ reinforces such inhibition by simultaneously reducing the pacemaker activity through the activation of inwardly rectifying K^+ channels and the inhibition of voltage-gated Ca^{2+} channels. If so, this could indicate the specificity of $\beta\gamma$ subunits in controlling the functions of effectors.

REFERENCES

1. Martin, T. F. (2003) *Biochim. Biophys. Acta* **1641,** 157–165
2. Rettig, J., and Neher, E. (2002) *Science* **298,** 781–785
3. An, S. J., and Almers, W. (2004) *Science* **306,** 1042–1046
4. Di Paolo, G., Moskowitz, H. S., Gipson, K., Wenk, M. R., Voronov, S., Obayashi, M., Flavell, R., Fitzsimonds, R. M., Ryan, T. A., and De Camilli, P. (2004) *Nature* **431,** 415–422
5. Felmy, F., Neher, E., and Schneggenburger, R. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15200–15205
6. Tse, A., Tse, F. W., Almers, W., and Hille, B. (1993) *Science* **260,** 82–84
7. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr. Rev.* **24,** 765–781
8. Stojilkovic, S. S., and Catt, K. J. (1995) *J. Neuroendocrinol.* **7,** 739–757
9. Ashworth, R., and Hinkle, P. M. (1996) *Endocrinology* **137,** 5205–5212
10. Bluet-Pajot, M. T., Epelbaum, J., Gourdji, D., Hammond, C., and Kordon, C. (1998) *Cell. Mol. Neurobiol.* **18,** 101–123
11. Freeman, M. E., Kanyicska, B., Lerant, A., and Nagy, G. (2000) *Physiol. Rev.* **80,** 1523–1631
12. Ben-Jonathan, N., and Hnasko, R. (2001) *Endocr. Rev.* **22,** 724–763
13. Sakaba, T., and Neher, E. (2003) *Nature* **424,** 775–778
14. Nagy, G., Reim, K., Matti, U., Brose, N., Binz, T., Rettig, J., Neher, E., and Sorensen, J. B. (2004) *Neuron* **41,** 417–429
15. Gillis, K. D., Mossner, R., and Neher, E. (1996) *Neuron* **16,** 1209–1220
16. Yang, Y., Udayasankar, S., Dunning, J., Chen, P., and Gillis, K. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 17060–17065
17. Zhu, H., Hille, B., and Xu, T. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 17055–17059
18. Grishanin, R. N., Kowalchyk, J. A., Klenchin, V. A., Ann, K., Earles, C. A., Chapman, E. R., Gerona, R. R., and Martin, T. F. (2004) *Neuron* **43,** 551–562
19. Silinsky, E. M. (1984) *J. Physiol.* **346,** 243–256
20. Silinsky, E. M., and Solsona, C. S. (1992) *J. Physiol.* **457,** 315–328
21. Blackmer, T., Larsen, E. C., Takahashi, M., Martin, T. F., Alford, S., and Hamm, H. E. (2001) *Science* **292,** 293–297
22. Van Goor, F., Zivadinovic, D., Martinez-Fuentes, A. J., and Stojilkovic, S. S. (2001) *J. Biol. Chem.* **276,** 33840–33846
23. Lachowicz, A., Van Goor, F., Katzur, A. C., Bonhomme, G., and Stojilkovic, S. S. (1997) *J. Biol. Chem.* **272,** 28308–28314
24. Tomic, M., Zivadinovic, D., Van Goor, F., Yuan, D., Koshimizu, T., and Stojilkovic, S. S. (1999) *J. Neurosci.* **19,** 7721–7731
25. Koshimizu, T. A., Tomic, M., Wong, A. O., Zivadinovic, D., and Stojilkovic, S. S. (2000) *Endocrinology* **141,** 4091–4099
26. Krsmanovic, L. Z., Mores, N., Navarro, C. E., Saeed, S. A., Arora, K. K., and Catt, K. J. (1998) *Endocrinology* **139,** 4037–4043
27. Serres, F., Li, Q., Garcia, F., Raap, D. K., Battaglia, G., Muma, N. A., and Van de Kar, L. D. (2000) *J. Neurosci.* **20,** 3095–3103
28. Sanchez-Blazquez, P., Garcia-Espana, A., and Garzon, J. (1995) *J. Pharmacol. Exp. Ther.* **275,** 1590–1596
29. Kedzierski, R. M., and Yanagisawa, M. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41,** 851–876
30. Lange, M., Pagotto, U., Renner, U., Arzberger, T., Oeckler, R., and Stalla, G. K. (2002) *Exp. Clin. Endocrinol. Diabetes* **110,** 103–112
31. Stojilkovic, S. S., Balla, T., Fukuda, S., Cesnjaj, M., Merelli, F., Krsmanovic, L. Z., and Catt, K. J. (1992) *Endocrinology* **130,** 465–474
32. Samson, W. K. (1992) *Biochem. Biophys. Res. Commun.* **187,** 590–595
33. Kanyicska, B., and Freeman, M. E. (1993) *Am. J. Physiol.* **265,** E601–E608
34. Harada, N., Himeno, A., Shigematsu, K., Sumikawa, K., and Niwa, M. (2002) *Cell. Mol. Neurobiol.* **22,** 207–226
35. Stojilkovic, S. S., Merelli, F., Iida, T., Krsmanovic, L. Z., and Catt, K. J. (1990) *Science* **248,** 1663–1666
36. Samson, W. K., Skala, K. D., Alexander, B. D., and Huang, F. L. (1990) *Biochem. Biophys. Res. Commun.* **169,** 737–743
37. Kanyicska, B., Burris, T. P., and Freeman, M. E. (1991) *Biochem. Biophys. Res. Commun.* **174,** 338–343

38. Stojilkovic, S. S., Iida, T., Merelli, F., and Catt, K. J. (1991) *Mol. Pharmacol.* **39,** 762–770
39. Tomic, M., Van Goor, F., He, M. L., Zivadinovic, D., and Stojilkovic, S. S. (2002) *Mol. Pharmacol.* **61,** 1329–1339
40. Zou, Y., Akazawa, H., Qin, Y., Sano, M., Takano, H., Minamino, T., Makita, N., Iwanaga, K., Zhu, W., Kudoh, S., Toko, H., Tamura, K., Kihara, M., Nagai, T., Fukamizu, A., Umemura, S., Iiri, T., Fujita, T., and Komuro, I. (2004) *Nat. Cell Biol.* **6,** 499–506
41. Zheng, L., Paik, W. Y., Cesnjaj, M., Balla, T., Tomic, M., Catt, K. J., and Stojilkovic, S. S. (1995) *Endocrinology* **136,** 1079–1088
42. Wong, Y. H., Conklin, B. R., and Bourne, H. R. (1992) *Science* **255,** 339–342
43. Fields, T. A., and Casey, P. J. (1995) *J. Biol. Chem.* **270,** 23119–23125
44. Wang, J., Frost, J. A., Cobb, M. H., and Ross, E. M. (1999) *J. Biol. Chem.* **274,** 31641–31647
45. Wickman, K. D., Iniguez-Lluhl, J. A., Davenport, P. A., Taussig, R., Krapivinsky, G. B., Linder, M. E., Gilman, A. G., and Clapham, D. E. (1994) *Nature* **368,** 255–257
46. Wolfe, J. T., Wang, H., Howard, J., Garrison, J. C., and Barrett, P. Q. (2003) *Nature* **424,** 209–213
47. Nagi, G., Matti, U., Nehring, R. B., Binz, T., Rettig, J., Neher, E., and Sorenson, J. B. (2002) *J. Neurosci.* **22,** 9278–9286
48. Blackmer, T., Larsen, E. C., Bartleson, C., Kowalchyk, J. A., Yoon, E.-J., Preininger, A. M., Alford, S., Hamm, H. E., and Martin, T. F. (2005) *Nat. Neurosci.* **8,** 421–425
49. Gerachshenko, T., Blackmer, T., Yoon, E.-J., Hamm, H. E., and Alford, S. (2005) *Nat. Neurosci.* **8,** 597–605